ICCVAM Test Method Evaluation Report: Current Validation Status of a Proposed *In Vitro* Testing Strategy for U.S. Environmental Protection Agency Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

2010

NIH Publication No. 10-7513

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This document is available electronically at:

http://iccvam.niehs.nih.gov/methods/ocutox/antimicro/TMER.htm

When referencing this document, please cite as follows:

ICCVAM. 2010. ICCVAM Test Method Evaluation Report: Current Validation Status of a Proposed *In Vitro* Testing Strategy for U.S. Environmental Protection Agency Ocular Hazard Classification and Labeling of Antimicrobial Cleaning Products. NIH Publication No. 10-7513. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

%CV	Percent coefficient of variation
AMCP	Antimicrobial cleaning product
ATWG	Alternative Testing Working Group
BCOP	Bovine corneal opacity and permeability
BRD	Background review document
CFR	Code of Federal Regulations
CM	Cytosensor Microphysiometer
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EO	EpiOcular™
EPA	U.S. Environmental Protection Agency
ET_{50}	Time needed to reduce cell viability by 50%
EU	European Union
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
HET-CAM	Hen's Egg Test Chorioallantoic Membrane
ICATM	International Cooperation on Alternative Test Methods
ICE	Isolated Chicken Eye
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IIVS	Institute for In Vitro Sciences, Inc.
ILS	Integrated Laboratory Systems, Inc.
IRE	Isolated Rabbit Eye
IVIS	*In vitro* irritancy score
JaCVAM	Japanese Center for the Validation of Alternative Methods
LVET	Low volume eye test
MRD_{50}	Estimated concentration of a test substance needed to reduce the basal metabolic rate of L929 cells by 50%
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NTP	National Toxicology Program
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development

OPP	Office of Pesticide Programs
OTWG	ICCVAM Ocular Toxicity Working Group
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SRD	Summary review document
TMER	Test method evaluation report
UN	United Nations
U.S.	United States
U.S.C.	United States Code

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Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions that submitted data to NICEATM for the evaluation of the antimicrobial cleaning product testing strategy.

Clorox Company
Pleasanton, CA

Colgate-Palmolive Company
Piscataway, NJ

The Dial Corporation
Scottsdale, AZ

EcoLabs
St. Paul, MN

Institute for In Vitro Sciences, Inc.
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Preface

Eye injury is a leading cause of visual impairment in the United States (U.S.) with 40,000 to 50,000 new cases of impaired vision reported each year.[1] Many eye injuries occur due to contact with workplace or household products or chemicals. Accidents involving common household products (e.g., oven cleaner and bleach) cause about 125,000 eye injuries each year.[2] These products often result in chemical burns and emergency room visits.[3] Each day about 2,000 U.S. workers have a job-related eye injury that requires medical treatment. Although the majority of these eye injuries result from mechanical sources, chemical burns from industrial chemicals or cleaning products are common.[4]

In order to avoid eye injuries, regulatory agencies require testing to determine if chemicals and products have the potential to cause eye damage. This testing information is used to classify the ocular hazard and to determine appropriate labeling that must be used to warn consumers and workers of the potential hazard and how to avoid exposures that could result in damage to the eye, and what emergency procedures should be followed if there is accidental exposure.

Nearly all ocular safety testing has been conducted using the Draize rabbit eye test, although *in vitro* methods can now be used to identify whether substances cause severe irritation or permanent eye damage. The Draize rabbit eye test, originally described by Draize et al. (1944), involves instillation of 0.1 mL of the test substance into the conjunctival sac of one eye while the other eye serves as the untreated control. The eye is examined at least daily for up to 21 days. The presence and severity of any injuries to the cornea, conjunctiva, and iris (tissues inside the eye) are scored and the duration that the injuries persist is recorded.

In 2004, the U.S. Environmental Protection Agency (EPA) requested that the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluate an *in vitro* testing strategy that would meet their need to evaluate, categorize, and label antimicrobial cleaning products (AMCPs) for eye irritation. As part of this evaluation, ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) requested the submission of data and information on AMCPs (73 FR 18535).[5]

ICCVAM carefully compiled and assessed all available data and arranged an independent scientific peer review. ICCVAM and its Ocular Toxicity Working Group (OTWG) solicited and considered public comments and stakeholder involvement throughout the evaluation process. As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) served as liaisons to the OTWG. ICCVAM, NICEATM, and the OTWG prepared a draft summary review document (SRD) describing the validation status of the AMCP testing strategy, including the reliability and accuracy of each of the three *in vitro* test methods in the AMCP testing strategy, and draft test method recommendations for usefulness and limitations. ICCVAM released this document to the public for comment on March 31, 2009, at which time ICCVAM also announced a meeting of the international independent scientific peer review panel (Panel) (74 FR 14556).[6]

The Panel met in public session on May 19–21, 2009, to review the ICCVAM draft AMCP SRD for completeness and accuracy. The Panel then evaluated (1) the extent to which the draft AMCP SRD addressed established validation and acceptance criteria and (2) the extent to which the draft AMCP

[1] Available at: http://www.preventblindness.org/resources/factsheets/Eye_Injuries_FS93.PDF
[2] Available at: http://www.geteyesmart.org/eyesmart/injuries/home.cfm
[3] From the CPSC NEISS Database, 2007.
[4] Available at: http://www.cdc.gov/niosh/topics/eye/
[5] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-6969.pdf
[6] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/E9-7220.pdf

SRD supported ICCVAM's draft test method recommendations. Before concluding their deliberations, the Panel considered written comments and comments made at the meeting by public stakeholders.

ICCVAM provided the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) with the draft AMCP SRD and draft test method recommendations, a summary of the conclusions and recommendations from the Panel meeting, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment. A detailed timeline of the evaluation is included with this report.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the test method evaluation process. ICCVAM considered the SACATM comments, the conclusions of the Panel, and all public comments before finalizing the ICCVAM test method recommendations for each test method. The recommendations and the SRD, which is provided as an appendix to this report, are incorporated in this ICCVAM test method evaluation report. As required by the ICCVAM Authorization Act, ICCVAM will forward its recommendations to U.S. Federal regulatory agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM–ICCVAM website,[7] and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. A. Wallace Hayes for serving as the Panel Chair and to Dr. Paul Bailey, Dr. Donald Sawyer, Dr. Kirk Tarlo, and Dr. Daniel Wilson for their service as Evaluation Group Chairs. We thank the OTWG for assuring a meaningful and comprehensive review. We especially thank Dr. Jill Merrill (U.S. Food and Drug Administration Center for Drug Evaluation and Research) and Dr. Karen Hamernik (EPA, to April 2009) for serving as Co-Chairs of the OTWG. Integrated Laboratory Systems, Inc., the NICEATM support contractor, provided excellent scientific support, for which we thank Dr. David Allen, Dr. Jonathan Hamm, Nelson Johnson, Dr. Brett Jones, Dr. Elizabeth Lipscomb, and James Truax. Finally, we thank European Centre for the Validation of Alternative Methods liaisons Dr. João Barroso, Dr. Thomas Cole, and Dr. Valerie Zuang and Japanese Center for the Validation of Alternative Methods liaison Dr. Hajime Kojima for their participation and contributions.

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[7] http://iccvam.niehs.nih.gov/methods/ocutox/AMCP.htm

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of the antimicrobial cleaning product (AMCP) testing strategy, including the performance of three *in vitro* test methods: bovine corneal opacity and permeability (BCOP), Cytosensor® Microphysiometer (CM), and EpiOcular™ (EO). This test method evaluation report (TMER) provides ICCVAM's recommendations regarding the usefulness and limitations of the AMCP testing strategy as well as recommendations for test method protocols, future studies, and performance standards. The report also includes ICCVAM's final summary review document (SRD) for the AMCP testing strategy.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and its Ocular Toxicity Working Group prepared the draft AMCP SRD and ICCVAM's draft test method recommendations. The drafts were provided to the public and an independent international scientific peer review panel (Panel) for comment. A detailed timeline of the ICCVAM evaluation process is appended to this report.

The Panel met in public session on May 19–21, 2009, to review and discuss the draft AMCP SRD and ICCVAM's draft test method recommendations. The Panel provided conclusions and recommendations on the validation status of the AMCP testing strategy. The Panel also reviewed how well the information contained in the draft SRD supported ICCVAM's draft test method recommendations. In finalizing this TMER and the AMCP SRD, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods, and (3) public comments.

Specific ICCVAM Recommendations

Test Method Usefulness and Limitations

Given that none of the 228 AMCPs in the validation database has been tested in all three *in vitro* test methods (i.e., BCOP, CM, and EO), ICCVAM concludes that the data are insufficient to adequately demonstrate that the AMCP testing strategy using these test methods can identify all four U.S. Environmental Protection Agency (EPA) ocular hazard categories.

ICCVAM also concludes that the data are insufficient to support definitive recommendations on the alternate AMCP testing strategy, which uses only the BCOP and EO test methods to classify substances in all four EPA ocular hazard categories. Only 28 of the 228 AMCPs have been tested in both the BCOP and EO test methods. Of these, the Draize rabbit eye test classified only one as an EPA Category II substance and only four as EPA Category III substances.

Test Method Protocol

ICCVAM recommends using the updated ICCVAM protocols for the BCOP, CM, and EO test methods that are included as appendices to this report. In addition, all future studies intended to further characterize the usefulness and limitations of these test methods (i.e., BCOP, CM, and EO) should be conducted using the ICCVAM recommended protocols.

Future Studies

Given the limitations of the validation database, ICCVAM recommends a reference list of AMCPs for which high-quality Draize rabbit eye test data are available. These AMCPs should then be tested in each of the proposed test methods (i.e., BCOP, CM, and EO) to more thoroughly evaluate their usefulness and limitations. ICCVAM recommends that future test methods consider cells and tissue constructs from ocular tissues. In addition, ICCVAM encourages industry stakeholders to provide strategies and approaches that are currently used for corporate decisions on product safety. Users

should make available data gathered from future studies. The data could be used to further characterize the usefulness and limitations of an *in vitro* testing strategy.

Performance Standards

ICCVAM concludes that the development of performance standards for the AMCP testing strategy is not warranted at this time.

Validation Status of the AMCP Test Methods and Testing Strategies

The Bovine Corneal Opacity and Permeability Test Method

The validation database included 66 substances tested in both the BCOP test method and the Draize rabbit eye test. The accuracy of the overall EPA classification (EPA Category I, II, III, or IV) was 55%. While the BCOP test method correctly classified only 60% of the EPA Category II and 50% of the EPA Category III substances, it correctly identified 90% of the EPA Category I substances.

Intralaboratory repeatability for the BCOP test method (i.e., comparison of within-experiment runs of a test substance) was determined for 67 AMCPs as the mean percent coefficient of variation (%CV) for opacity (21%), permeability (25%), and *in vitro* irritancy score (IVIS) (18%). NICEATM also evaluated agreement with the ocular hazard classifications of the EPA and the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The EPA and GHS classification systems had 100% agreement in 63 of the 75 test runs (84%), 67% agreement in 11 of the 75 test runs (15%), and 60% agreement in one of the 75 test runs (1%).

NICEATM determined intralaboratory reproducibility (i.e., comparison of between-experiment runs of a test substance) for five AMCPs as the mean %CV for the IVIS. In two to six experiments, the mean %CV for the IVIS was 20%. These test substances were also evaluated for their agreement with the EPA and GHS ocular hazard classification systems. The evaluation found 100% agreement among the five test substances.

Interlaboratory reproducibility (i.e., comparison of runs of a test substance between different laboratories) for the BCOP test method could not be determined specifically for AMCPs because only one laboratory conducted the testing. However, three studies (3-12 laboratories each) determined interlaboratory reproducibility for non-AMCPs classified as severe or ocular corrosives by the BCOP test method (ICCVAM 2006a). The mean %CV for IVIS ranged from 25% to 36%.

The Cytosensor Microphysiometer Test Method

The validation database included 105 unique substances tested in both the CM test method and the low volume eye test (LVET). Three substances were tested twice for a total of 108 substances. The accuracy of the overall EPA classification (i.e., EPA Category I, II, III, or IV) was 30%. All nine of the EPA Category I substances were correctly identified. The CM test method overclassified the majority of substances classified by the LVET as EPA Category II, III, or IV substances (100% of the Category II substances, 67% of the Category III substances, and 89% of the Category IV substances).

Reliability of the CM test method could not be evaluated specifically for AMCPs due to insufficient data. However, NICEATM evaluated reliability of the test method in non-AMCPs. Intralaboratory repeatability was evaluated based on data from seven different studies of 1 to 35 substances. The mean %CV for the concentrations needed to reduce the basal metabolic rate of L929 cells by 50% (MRD_{50} values) ranged from 6% to 25% for all materials tested.

Intralaboratory reproducibility for the CM test method was determined for 16 non-AMCP substances in one laboratory. The mean %CV for MRD_{50} values for all substances tested was 25%.

Interlaboratory reproducibility for the CM test method was measured using results from two studies at two to four laboratories each. The mean %CV for MRD_{50} values for all substances tested ranged from 17% to 51%.

The EpiOcular™ Test Method

Thirty substances were tested in both the EO test method and the Draize rabbit eye test. The accuracy of the overall EPA classification (i.e., EPA Category I, II, II, or IV) was 76%. All of the EPA Category I substances were correctly identified. Of the four EPA Category III substances, 75% were correctly identified by the EO test method. Forty-four percent of the nine EPA Category IV substances were correctly identified.

NICEATM determined intralaboratory repeatability for the EO test method in a subset of 15 AMCPs. The mean %CV for the times needed to reduce cell viability by 50% (ET_{50} values) ranged from 0% to 62%. To evaluate the extent of agreement between the EPA and GHS ocular hazard classification systems, NICEATM analyzed intralaboratory reproducibility for three AMCPs that had been tested more than once at one laboratory. The three AMCPs had 100% agreement in both EPA and GHS classification systems. Intralaboratory reproducibility for the EO test method was also determined from repeat testing of a single substance (0.3% Triton X-100), which occurred at two different laboratories. The mean %CV for ET_{50} values was approximately 20% in both laboratories.

Interlaboratory reproducibility for the EO test method was determined for non-AMCPs in a two-phase validation study of surfactants and surfactant-containing products. Mean %CVs ranged from 12% to 18%. However, this evaluation did not use a calculated ET_{50} value to predict the ocular hazard classification category, as detailed in the AMCP background review document. Instead, it was based on an EO protocol that uses relative percent viability to classify irritancy (i.e., irritant vs. nonirritant).

These test substances were also evaluated for agreement in the EPA and GHS ocular hazard classification systems. Using either the EPA or GHS classification system in one phase of the validation study, 74% of the 19 substances had 100% agreement. In a subsequent study phase, 94% of the 54 substances had 100% agreement.

Original Testing Strategy Proposed in the AMCP Background Review Document: Combining the BCOP, CM, and EO Test Methods

The AMCP testing strategy (**Figure 1**) uses three *in vitro* test methods: BCOP, CM, and EO. For each test method, decision criteria have been developed to correspond to the four different categories of ocular irritation defined by the EPA hazard classification system.

The first test method used in the AMCP testing strategy depends on the chemical properties of the test substance. If the test substance is an oxidizer, which suggests that it will be an ocular corrosive or severe irritant, then the BCOP test method is used first. Test substances that produce an IVIS $\geq$ 75 in the BCOP would be classified as EPA Category I. If a test substance produces an IVIS < 75, further assessment using histopathology evaluation of the affected tissue can then determine whether it meets the criteria for classification as EPA Category I, II, or III.

To determine whether the test substance is EPA Category III or IV, the test substance is subsequently tested in either the CM or EO test method. The choice of method again depends on the chemical properties of the test substance. If the test substance is water soluble, it can be tested in either the CM test method or the EO test method. If it is water insoluble, it must be tested in the EO test method to determine the final hazard classification.

None of the 228 substances in the validation database has been tested in all three of the *in vitro* test methods proposed for the AMCP testing strategy. Therefore, no data are available with which to characterize the actual performance of a testing strategy that includes the BCOP, CM, and EO test methods.

Alternate AMCP Testing Strategy: Combining the BCOP and EO Test Methods

As explained above, none of the 228 AMCPs included in the original testing strategy has been tested in all three of the *in vitro* test methods included in the AMCP testing strategy. However,

28 substances were tested in both the BCOP and EO test methods. ICCVAM also had concerns about the validation status of the low volume eye test, which was used as the *in vivo* reference test method for all of the CM test method data. Therefore, ICCVAM evaluated an alternate AMCP testing strategy (**Figure 2**) that included only the BCOP and EO test methods. The alternate AMCP testing strategy was evaluated using two approaches: (1) test in the BCOP test method first to identify EPA Category I and II substances and then test in the EO test method to identify EPA Category III and IV substances; or (2) test in the EO test method first to identify EPA Category III and IV substances and then in the BCOP test method to identify EPA Category I and II substances.

The alternate AMCP testing strategy performed the same regardless of which approach was used. It correctly classified 79% of the substances, identifying 100% of the EPA Category I substances, none of the EPA Category II substances, 100% of the EPA Category III substances, and 44% of the EPA Category IV substances.

Figure 1 Combining the BCOP, CM, and EO Test Methods into a Testing Strategy: AMCP Testing Strategy

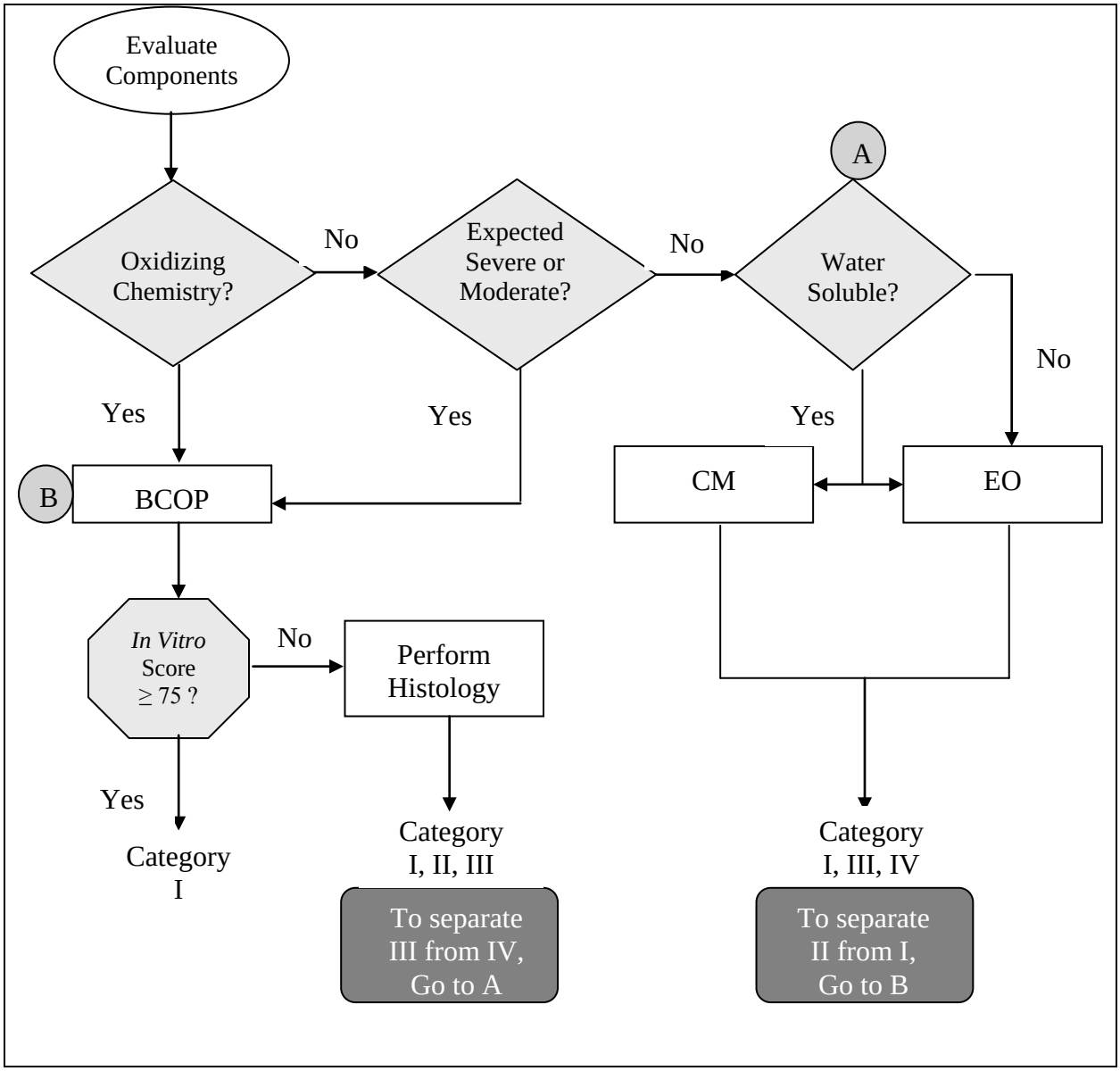
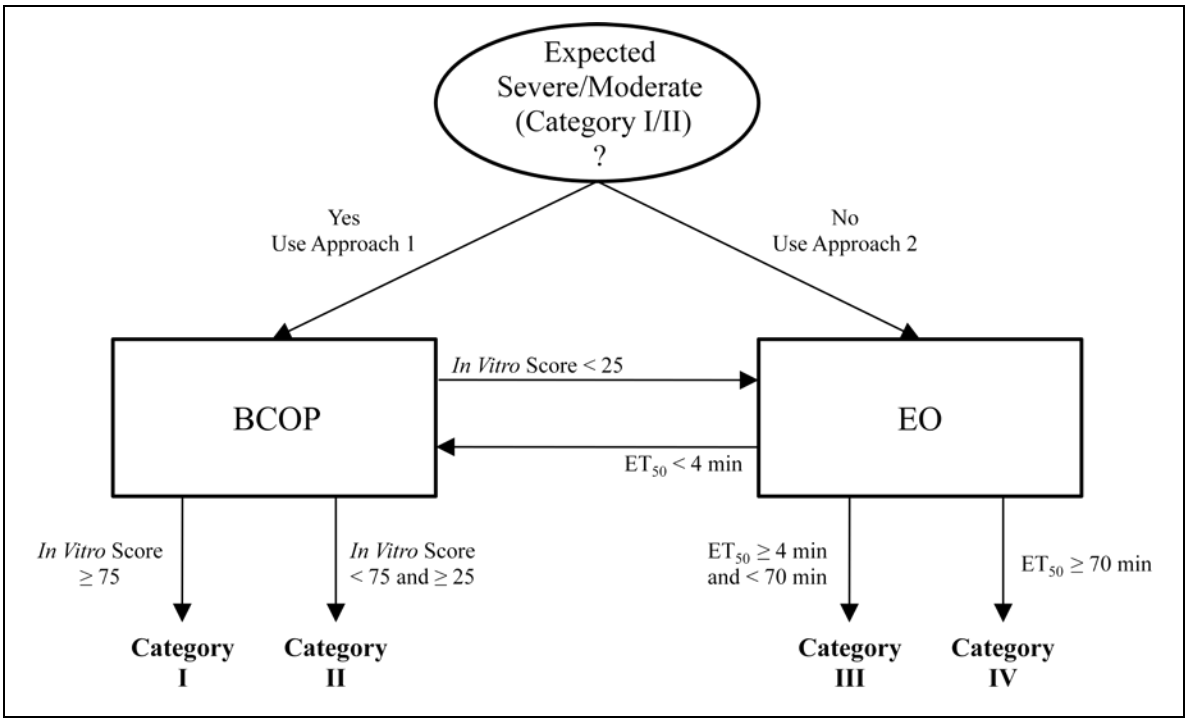


Figure 2 Combining the BCOP and EO Test Methods into a Testing Strategy: Alternate AMCP Testing Strategy



ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement. The public may submit written comments and provide oral comments at ICCVAM independent peer review panel meetings and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) meetings. From March 2005 to July 2009, there were nine opportunities for public comment on ICCVAM's evaluation of the validation status of alternative ocular safety testing methods and approaches. During this time, ICCVAM received 37 public comments, of which 25 pertained directly to the AMCP testing strategy or one of the three *in vitro* test methods (i.e., BCOP, CM, and EO) included in the AMCP testing strategy. SACATM reviewed and commented on the draft recommendations and associated conclusions of the Panel during their annual meeting in June 2009. ICCVAM considered public and SACATM comments in finalizing the test method recommendations provided in this report.